Antigen Markers for Clinical Manifestations and Prevention of HTLV-III/LAV Infections

Annual Report

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February 25, 1989

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7031

Harvard University School of Public Health
Boston, MA 02115

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp. Date Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Harvard University School of Public Health 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 665 Huntington Avenue Boston, MA 02115	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION US Army Medical Research and Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-87-C-7031
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 6310 5A PROJECT NO 3M2-63105DH29 TASK NO. AB WORK UNIT ACCESSION NO 046

11. TITLE (Include Security Classification)

Antigen Markers for Clinical Manifestation and Prevention of HTLV-III/LAV Infections

12. PERSONAL AUTHOR(S)
Tun-Hou Lee

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 1/26/88 TO 1/25/89	1989 February 25	27 pages

16. SUPPLEMENTARY NOTATION

17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	03		HIV, AIDS, gp120, RAI
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

During this year of our research we looked further at the immune response to various HIV-1 antigens, concentrating especially on the response to the envelope glycoprotein gp120. Using serum samples from hemophiliacs we found that the first antibodies detectable by Western blot are those against gp120 (NR) domains--those which are conformationally-dependent and which are not detected under reducing conditions. This suggests that the reducing conditions used in most Western blot confirmatory tests miss detection of these antibodies. Since these gp120 (NR) antibodies persist throughout the course of infection at high titers, it seems that they are very immunogenic, but perhaps not very protective against the virus. We also found a significantly higher rate of progression to AIDS-related complex (ARC) for individuals lacking antibodies to gp120(R). In studies focussing on HIV-2 and SIV, we identified a new gene, now called *vpx*, which is not found in the genome of HIV-1. The *vpx* product is a protein of about 12 kd in SIV and 16 kd in HIV-2 which is virion-associated, but does not appear to be necessary for the *in vitro* replication of SIVmac. Since *vpx* is not present in HIV-1, it may be used to distinguish HIV-1 from HIV-2 infections.

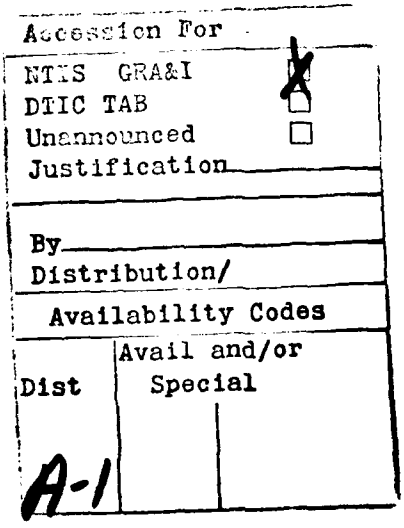
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	21 ABSTRACT SECURITY CLASSIFICATION unclassified
22a NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian	22b TELEPHONE (Include Area Code) 301-663-7325 22c OFFICE SYMBOL SGRD-RMI-S

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.



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Summary

The major objective of the proposed study is to identify HIV gene products and to determine if antibodies elicited by these products can be used to predict the clinical outcome of HIV infection. During the first year of our research we conducted studies in which we located a new gene in the central region of the HIV-1 genome. This gene encodes a 16 kd protein which is unique to HIV-1 and does not appear in HIV-2. We also performed seroepidemiological studies and identified a domain located in the C-terminal third of gp120 [gp120(R)], whose immunogenicity is preserved even under reducing conditions, and is therefore not conformationally-dependent. Antibodies to this portion of gp120 are more prevalent in individuals in the early stages of disease, and in seropositive individuals who are less likely to progress to AIDS than those with low levels of antibodies against gp120(R).

During the second year of our research we looked further at the immune response to various HIV-1 antigens, concentrating especially on the response to the envelope glycoprotein gp120. Using serum samples from hemophiliacs we found that the first antibodies detectable by Western blot are those against gp120(NR) domains--those which are conformationally-dependent and which are not detected under reducing conditions. This suggests that the reducing conditions used in most Western blot confirmatory tests miss detection of these antibodies. Since these gp120(NR) antibodies persist throughout the course of infection at high titers, it seems that they are very immunogenic, but perhaps not very protective against the virus. We also found a significantly higher rate of progression to AIDS-related complex (ARC) for individuals lacking antibodies to gp120(R). In studies focussing on HIV-2 and SIV, we identified a new gene, now called *vpx*, which is not found in the genome of HIV-1. The *vpx* product is a protein of about 12 kd in SIV and 16 kd in HIV-2, which is virion-associated, but does not appear to be necessary for the *in vitro* replication of SIVmac. Since *vpx* is not present in HIV-1, it may be used to distinguish HIV-1 from HIV-2 infections.

Section 1: Antibody Responses in Early Human Immunodeficiency Virus Type 1 Infection

At the beginning of the AIDS epidemic we were limited in our research efforts by our inability to look retrospectively at the course of HIV infection. Now, although still thwarted somewhat by the tremendous lag time between infection and the onset of disease symptoms, we are better able to obtain past serum samples from patients with known outcomes. Sequential analysis of these patient sera is invaluable in determining the profiles of human anti-HIV antibodies which develop and change over the course of infection. Knowledge of these profiles is important for two reasons: First, it not only helps to verify human reactivity to various HIV-1 antigens, but it also helps to determine which specific antibodies are most likely to be observed at which times over the course of HIV infection. And second, observed correlations between specific antibody responses and any given clinical status might help to determine which antigens should be evaluated for their effective use in a vaccine against AIDS.

This report describes results from seroepidemiologic studies using a total of 357 sequential serum samples from 201 Greek hemophiliacs, 98 (49%) of whom were in the early stages of HIV infection. Of these seropositive individuals, 8 had been diagnosed as having ARC (1).

General Trends in Antibody Development

Each serum sample was tested for reactivity against 7 HIV-1 specific antigens, 5 from the *env* gene and 1 each from *gag* and *pol*. Of the env gene antigens, 1 represented gp160, 1 gp41, and the remaining 3 were different preparations of gp120-- (i) preparation from cell lysate for radioimmunoprecipitation (RIP), (ii) from viral lysate for WB under reducing conditions [gp120(R)], and (iii) from viral lysate for Western blotting (WB) under non-reducing conditions [gp120(NR)]. Eight combinations of antibodies directed to the seven antigens were obtained by analyzing WB and RIP antibody profiles of the 215 positive specimens (Table 1). Three-fourths of the positive specimens contained antibodies to all of the seven antigens. In general, after analyzing 157 positive serial samples from 43 individuals, neither a decrease nor a loss of specific

reactivity to any of the seven antigens was observed. The progressive gain of antibody reactivity to some antigens, where the patient began with pattern 1, 2, 3, 4, or 5 and progressed to pattern 8 suggested a general pattern of antibody development (Fig. 1). Detectable antibodies probably develop earliest to gp160 and to gp120(RIP), followed by antibodies to p24, gp120(NR), and p64, then by antibodies to gp41, and finally by antibodies to gp120(R) (Table 1). This shows that antibodies to two antigens, p24 and gp41, that are currently used to judge seropositivity via the WB technique are not necessarily the most frequently detected antibodies.

Antibody Response to Different Preparations of gp120

The gp120 antigen was defined on the basis of three classes of antigenic reactivity: gp120(RIP), gp120(NR), and gp120(R) (1,2). Among the three, gp120(RIP) was the most consistently positive for detecting antibodies. Antibodies to gp120(RIP) appeared in all of the positive specimens. The relative efficiency of gp120(NR) was slightly less than gp120(RIP). However, when the reducing agent dithiothreitol was incorporated into the sample buffer, the resulting antigen, [gp120(R)], was the least efficient, not only among the three gp120 antigens studied, but also among all of the seven antigens analyzed in the present study. Thus, antibodies to gp120 were undetectable in 25.6% of the samples that actually contained such antibodies when the reduced form of HIV lysate [gp120(R)] was used as the only source of antigen. The reduced immunogenicity of gp120(R) as compared with gp120(RIP) or gp120(NR) suggests that disulfide linkages between cysteine residues play a significant role in maintaining the configurational integrity of gp120(NR). Nevertheless, the presence of antibody to gp120(R) also indicates that the disruption of disulfide bonds does not completely destroy the antigenicity of gp120 (1).

The Relative Immunogenicities of gp120(R) and gp120(NR)

Several lines of evidence suggest that gp120(NR) epitopes are more immunogenic than gp120(R) epitopes (3). First, antibodies directed to gp120(NR) epitopes are produced before those directed to gp120(R) epitopes. In addition, previous analyses of HIV-1 seropositive patients revealed that patients who were at later stages of HIV-1 infection lacked detectable gp120 antibodies directed to gp120(R) epitopes more often than they lacked those to gp120(NR). In fact,

antibodies directed to gp120(NR) were readily detected in all seropositive patients studied (1). Indeed, our results from the hemophiliac study indicated that all seropositive patients had gp120(NR) antibodies, while half of the AIDS-related complex (ARC) patients lacked detectable gp120(R) antibodies. These findings suggest that the prevalence of gp120(NR) antibodies is higher than that of gp120(R) antibodies in HIV-1 seropositive patients who are at later stages of infection.

Immune Protection Afforded by Antibodies against Specific gp120 Epitopes

The titer of natural gp120 antibodies that block gp120-CD4 interaction or neutralize cell-free virions is at least 3 to 4 logs lower than the titer of gp120-binding antibodies. One of the likely explanations for this difference is that the majorty of gp120 antibodies produced during natural infection are directed to regions that are immunologically dominant, but functionally unimportant (3,4). Analysis of sequential serum samples indicates that during disease progression gp120(NR) antibodies are persistently present. These observations not only demonstrate that antibodies elicited by the immunologically more dominant gp120(NR) epitopes can persist throughout the course of disease, but they also raise the question as to whether or not gp120(NR) antibodies are protective. Indeed, it seems that the immunodominant gp120(NR) epitopes may induce antibodies that are largely non-protective. It is conceivable that gp120(NR) epitopes may serve as decoys to draw antibody response away from other gp120 epitopes. It is also possible that gp120(NR) antibodies may interfere with neutralizing antibodies via steric hinderance.

Association between the Presence of Antibodies against gp120(R) and Disease Progression

We noted an association between an absence of antibodies to gp120(R) and the development of clinical symptoms. Among the six hemophiliacs who did not demonstrate sero-reactivity to this antigen in the last available positive serial specimens, four have already developed ARC. The rate ratio of clinical progression to ARC was as high as 6.2 (95% confidence interval, 2.1-18.3, p=.0011) when those hemophiliacs who did not have this seroreactivity in the last positive serial specimen were compared with those who did have such antibodies. The strong association between the absence of antibody to gp120(R) and clinical progression to ARC (Table

2) suggests a possible prognostic value for tests that include an evaluation of levels of antibody to gp120(R). The role that antigenic epitopes represented by gp120(R) may play in the pathogenesis of infection may be important for evaluating prospects for HIV immunoprophylaxis.

Conclusions

In early HIV-1 infection, antibodies to viral antigens seem to increase gradually, with antibodies against the envelope gene product gp120(RIP) as the first detectable antibody. In contrast to findings with late-stage patients, in early infection, there does not seem to be a loss of antibodies to any of the tested antigens. In addition, there are epitopes of gp120 that maintain their immunoreactivity even under reducing conditions in which disulfide linkages between cysteine residues are destroyed. These epitopes are less immunogenic than the gp120(NR) epitopes which are conformationally-dependent, but they may in fact be more valuable for protection against HIV than are the more immunodominant epitopes.

Because the most commonly used serologic confirmatory tests use viral antigens treated with a reducing buffer, antibodies to the gp120(NR) epitopes not detected. The earlier appearance of the antibody to gp120(NR), as compared with gp41, suggests that gp120(NR) may be an important antigen for evaluating seropositivity by WB. To make use of this reactivity, however, it is necessary to maintain the conformational integrity of gp120 and to avoid the use of reducing agents when preparing the viral antigen.

Materials and Methods

Sera. Two hundred one Greek hemophiliacs from the Hemophilia Center of the Second Regional Blood Transfusion Center (Athens, Greece) were studied. Single or up to 11 serial serum samples from 52 people were collected between September 1981 and July 1986. The serum samples were transmitted to Boston by air and stored at -20°C until used. A total of 357 serum samples, including 149 (41.7%) single specimens, were analyzed for the present study.

Serological assays. Serum samples were tested for antibodies to HIV-1 by three procedures: RIP, WB with nonreducing sample buffer (NR), and WB with reducing sample buffer (R). A positive reference serum from an American homosexual man with AIDS-related

complex (ARC) and a negative reference serum from a laboratory worker were included with each test.

Radioimmunoprecipitation. As previously described for RIP (5), HTLV-IIIB infected Molt-3 cells were metabolically labeled with ^{35}S-cysteine and ^{35}S-methionine (New England Nuclear, Boston) disrupted with RIPA buffer (0.15M NaCl, 0.05 M Tris–HCl [pH 7.2], 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), and then centrifuged for 1 h at 100,000 g. The lysate supernatant was then reacted with each serum sample after being readsorbed with protein A-Sepharose CL-4B (Sigma, St. Louis). Immunoprecipitates were eluted in sample buffer containing 0.1 M Cleland's reagent (dithiothreitol), 2% SDS, 0.08 M Tris-HCl (pH 6.8), 10% glycerol, and 0.2% bromophenol blue by boiling for 2 min. Samples were analyzed in the discontinous buffer system of Laemmli (6) with 13.1% acrylamide resolving gels and 3.0% stacking gels.

Western blotting. For WB (7), cell-free supernatant obtained from the culture fluid of Molt-3 cells persistently infected with HTLV-IIIB was filtered through a 0.45 mm membrane and ultracentrifuged at 20,000 g for 2 h with 20% sucrose in PBS as cushion. The virus pellet was then lysed in RIPA buffer, and then mixed either with twofold-concentrated reducing sample buffer as described for RIP analysis or with nonreducing sample buffer lacking the reducing agent dithiothreitol. This mixture was then boiled for 2 min and fractionated by SDS-PAGE. The resulting gel was washed in transfer buffer (10mM Tris-HCl [pH 7.0], 2mM EDTA, 50mM NaCl), and the proteins were passively transferred to 0.45 mm nitrocellulose sheets (Schleicher and Schuell, Keene, NH) for approx. 36 h at room temperature (approx. 23 C). The nitrocellulose sheets were then incubated at 37 C with 3% bovine serum albumim (BSA) in PBS, washed with wash buffer (0.2% Tween 20 in PBS), and cut into 0.5 cm strips. Each strip was individually laid into a trough containing dilution buffer (1% BSA in wash buffer) in an incubation tray (Bio-Rad, Richmond, Calif). A single serum sample was then added to each trough and incubated at room temperature for 2 h. The strips were then washed with wash buffer and incubated with a 1:500 dilution of biotin-conjugated, affinity-purified antibody to human immunoglobulin (Amersham,

Arlington Heights, Ill) for 1 h at 37 C, washed again, and incubated with a 1:500 dilution of streptavidin-biotinylated horseradish peroxidase complex (Amersham) for 1 h at 37 C. After washing with wash buffer and PBS, peroxidase activity was detected by a reaction with freshly prepared 0.05% (wt/vol) of 3,3'–diaminobenzidine tetrahydrochloride and 0.05% of 30% H_2O_2 in PBS.

Section 2: A Naturally Immunogenic Virion-Associated Protein Specific for HIV-2 and SIV

HIV-1 and -2 and SIV have all been shown to have extraordinarily complex genomes. In addition to the traditional retroviral genes, gag, pol and env, these viruses also have numerous accessory or regulatory genes (8-15). We recently identified a gene, now called vpu (16), that occurs in HIV-1 but not HIV-2, and it was thought that the presence of this gene might be useful in distinguishing infections with these two virus types. We have now identified a second gene, now called vpx, which occurs in HIV-2 and SIV but not in HIV-1. The gene is located in an open reading frame of the SIV and HIV-2 genomes which was formerly called orf-x . The product of this gene induces an antibody response in both humans and monkeys and should also be valuable in distinguishing HIV-1 infection from HIV-2 infection.

Figure 2 shows the construction of plasmid pX-2 which was engineered to produce recombinant SIVmac vpx encoded peptides when transfected into E. coli. This vector is modified from plasmids pUC-18 and pXVR (17), and contains a 3.1 kb Stu I- Pst I fragment from an SIV infectious clone pBK-28 (18-19). The vpx sequence in pX-2 was determined to be in-frame with the preceding v-ras^H sequence by DNA sequence analysis (Figure 2b). Upon induction by isopropyl-ß-D-thiogalatopyranoside (IPTG), a v-ras^H/vpx fusion protein of about 27 K was detected (Figure 2c). This protein was recognized by goat antibodies to v-ras^H encoded p21 and it was not detected in uninduced cells.

We were able to show that this gene produces a functional protein product because it reacted with both human and monkey sera (Figure 3). Antibody reactivity to the recombinant vpx protein was detected in 35 of the 42 HIV-2 seropositive West Africans. In contrast with the high antibody prevalence among HIV-2 infected people, only 11 of the 52 SIV-seropositive monkeys studied had detectable antibody reactivity to this protein. None of the 41 HIV-1 antibody positive human sera from U.S. and West African donors nor the 31 U.S. and West African blood donor sera that were negative for antibodies to HIV-1 and HIV-2 reacted with this protein. The native protein product was identified for SIV by radioimmunoprecipitation in which post, but not pre-immune goat serum

against the recombinant vpx protein detected a 12 kd protein in SIVmac-infected Hut-78 cells (Figure 4a). This protein ($p12^x$) was also cross-reactive with human HIV-2-positive sera.

It was then found that the vpx-encoded protein was virion-associated since it could be detected in viral lysates by immunoblotting (Figure 4a,b). $P12^x$ was specifically recognized by an SIV seropositive monkey serum and by post-immune goat serum directed to the recombinant vpx protein. It was not recognized by pre-immune goat serum, or by SIV seronegative monkey serum.

The native vpx product in the HIV-2 virus was identified by immunoblotting in viral lysates prepared from $HIV\text{-}2_{SBL\text{-}6669}$ producer cells (19-20). A protein of about 16 kd was specifically recognized by post-immune goat serum directed to the SIV_{MAC} vpx recombinant protein, but not by pre-immune goat serum (Figure 4c). Likewise, this 16 kd protein product was recognized by HIV-2 positive human sera, but not by HIV-1 positive human sera or human sera negative for both HIV-1 and HIV-2. Cross-reactivity to the $HIV\text{-}2_{SBL\text{-}6669}$ vpx protein by SIV seropositive monkey sera was also observed.

We also compared the replication and infectivity of wild-type SIV viruses and mutants with truncated vpx products. According to immunoblotting analysis, we did not find a significant difference in the amount of virion proteins produced by cells infected with the wild type virus and the vpx mutant virus at 9 days post-infection. However, at 4 days post-infection the wild type virus infected cells appeared to produce fewer virions (Figure 5). This observation is compatible with the hypothesis that the vpx gene has a negative, and therefore dispensable, role in the replication cycle of SIV_{MAC}.

Conclusion

The open reading frame formerly called orf-x, is a gene, now called vpx, which is capable of producing a functional protein product. This gene is present only in HIV-2 and SIV and not in HIV-1, and its product appears as a 12 kd protein in SIV and a 16kd protein in HIV-2. It seems to be virion-associated and is cross-reactive between strains of SIV and HIV-2. Its exact function is still unclear, but it does seem to be dispensable for efficient replication and cell-free infectivity

functions in SIVmac. Some evidence suggests that it may play a role in the negative regulation of these viruses.

Materials and Methods

Plasmid pX-2.

This plsmid is modified from plasmid pUC-18 and contains part of plasmids pXVR (17) and pBK-28(18-19). It contains an EcoR I fragment which has a tac promoter linked to the first 333 nucleotides of v-ras^{H}. Downstream from the v-ras^{H} coding sequence is the pUC-18 polylinker cloning site. The 3.5 kb Sst I fragment of pBK-28 was excised and subcloned into pUC-18. This subcloned plasmid was digested with Stu I and ligated with a Bam HI linker, d(CGGGATCCCG). Plasmid DNA with the Bam HI linker was digested with Bam HI and Pst I. The 3.1 kb Bam HI-Pst I fragment which includes most of the vpx coding sequence was inserted into the polylinker cloning site.

DNA sequence analysis of pX-2.

The nucleotide sequence of a 0.6 kb Hind III DNA fragment beginning in the v-ras^{H} gene and ending in the R region of pBK-28 was determined by the dideoxy nucleotide sequencing method (USB, Cleveland, Ohio).

Polyacrylamide gel electrophoresis of recombinant vpx protein.

Whole bacterial lysates were prepared as follows: 200 ul of an overnight culture of X-90 (22) bearing pX-2 was inoculated into 10ml Luria-Bertani media containing 50 ug/ml ampicillin, and the cells were grown to an optical density of 0.3 at 550 nm. 5 ml cultures were induced with 5 mM IPTG and the remaining volume was used as an uninduced control. After 3h at 37^{o} C, the cells were collected by centrifugation at 3000 rpm (Beckman AccuSpin FR) for 10 min and washed once with 50 mM NaCl, 10 mM Tris-Cl pH 7.5. Cells were lysed in 500 ul of Laemmli sample buffer (6) and boiled for 2 min before loading onto the gel. Partial purification procedure for vpx recombinant protein was modified from that of Pallas et al. (22). All the steps were the same except that the final wash was carried out in 8 M urea. For each 250 ml bacterial culture 6 ml of 8

M urea buffer was used. Samples were analyzed on 15% polyacrylamide gels using the Laemmli buffer system (6) and stained with Coomassie blue.

Immunoblotting of partially purified vpx recombinant protein.

Procedures for immunoblotting were as described (7). Goat anti-ras serum was raised against a recombinant protein containing the first 111 amino acid residues of v-ras^H. Approximately 0.8 mg of polyacrylamide gel purified protein was used for initial immunization. Booster shots were given on days 14 and 21 with approximately 0.4 mg of the same material. A serum collected 28 days post-immunization was used in this study. Procedures for partial purification of vpx recombinant protein were followed for purification of the ras protein expressed by plasmid pXVR. All HIV-2 positive sera were found to have more binding reactivity to the env gene encoded gp120 of SIV_{MAC} than to that of $HIV\text{-}1_{IIIB}$ (M.J. Chou, unpublished data).

Detection of $p12^x$ in SIV_{MAC} infected Hut-78 cells by radioimmunoprecipitation (RIP)

Cell lysates were prepared from [^{35}S]cysteine labelled Hut-78 cells infected with wild type virus or the vpx mutant virus. Details of the RIP/SDS-PAGE techniques have been described (23). Viral lysates were prepared as follows: 60 ml of cell-free supernatants from the wild type virus or the vpx mutant virus infected Hut-78 cells were filtered through 0.45 micron filter units and centrifuged at 20,000 rpm (Beckman SW 28 rotor) for 2h through a 20% (w/v) sucrose cushion. Virus pellets were lysed in 200 ul RIPA lysis buffer (23) and subject to immunoblotting (7). To generate post-immune goat serum to the vpx recombinant protein, approximately 0.8 mg of polyacrylamide gel purified protein was used in the first immunization. Booster shots were given on days 14 and 21 with approximately 0.4mg of the same material . A serum collected 28 days post-immunization was used in this study.

Detection of $p12^x$ in sucrose gradient banded SIV_{MAC}.

Virions prepared from the cell-free culture supernant of wild type virus infected Hut-78 cells were layered on 20%-50% (W/V) sucrose gradients and centrifuged at 35,000 rpm (Beckman SW41 rotor) for 6 hours. One mililiter fractions were obtained dropwise from the bottoms of the tubes. A reverse transcriptase assay (24) was performed on a 72 microliter portion of each fraction.

Fractions 4 and 5 were pooled and centrifuged at 35,000 rpm (Beckman SW41 rotor) for one hour. Procedures for the preparation of viral lysates and immunoblotting were as described above.

Immunoblotting of HIV-2$_{SBL-6669}$ vpx protein.

30 ml cell-free supernatants from HIV-2$_{SBL-6669}$ producer cells were used for immunoblotting. Procedures for the preparation of viral lysates and immunoblotting were as described above.

Immunoblotting of virion proteins produced by the wild type virus or the vpx mutant virus infected Hut-78 cells.

Five million Hut-78 cells were transfected with 5ug of pBK-28 or pBK-x by the DEAE - dextran method (18). Cell-free supernantants collected 9 days post-transfection were used to infect fresh Hut-78 cells. Procedures for the preparation of viral lysates and immunoblotting were as previously described. Prestained molecular weight markers were from Amersham (Arlington Heights, IL).

Table 1. Reactivity patterns to HIV-1 antigens for antibody positive serum specimens from Greek hemophiliacs.

Pattern no.	HIV-1 antigens					
	p24	gp160 of sera	gp120(RIP)	gp120(NR)	gp120(R)	gp41	p64
1	+	+*	–	–	–	–
2	+	+	–	–	–	–
3	+	+	+	–	–	–
4	+	+	+	–	–	+
5	+	+	+	–	+	+
6	+	+	+	–	+	+
7	+	+	+	+	–	+
8	+	+	+	+	+	+
Total						

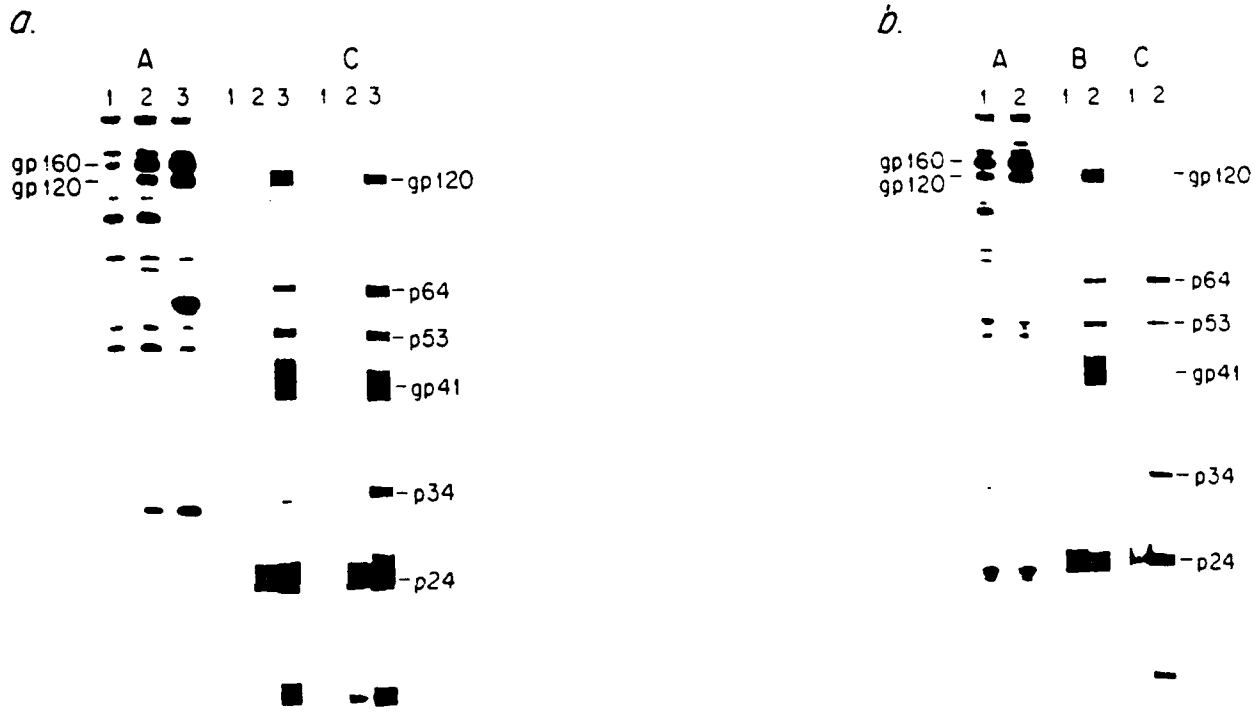
* The reactivity was observed when viral lysate was used, although reactivity was only seen to gp160 when cellular lysate was used (figure 1, panel a, lane 1).

Table 2. Association between the inability to respond to gp120(R) and the progression to ARC.

Subjects	Antibody to gp120(R)		
	Absence	Presence	Total
With ARC	4	4	8
Healthy	9	81	90
Total	13	85	98

NOTE. Data are no. of subjects. Rate ratio = 6.5, 95% confidence interval = 2.5-17.4, X^2 = 10.22 and P = .0014 for subjects with vs. without antibody.

Figure 1.

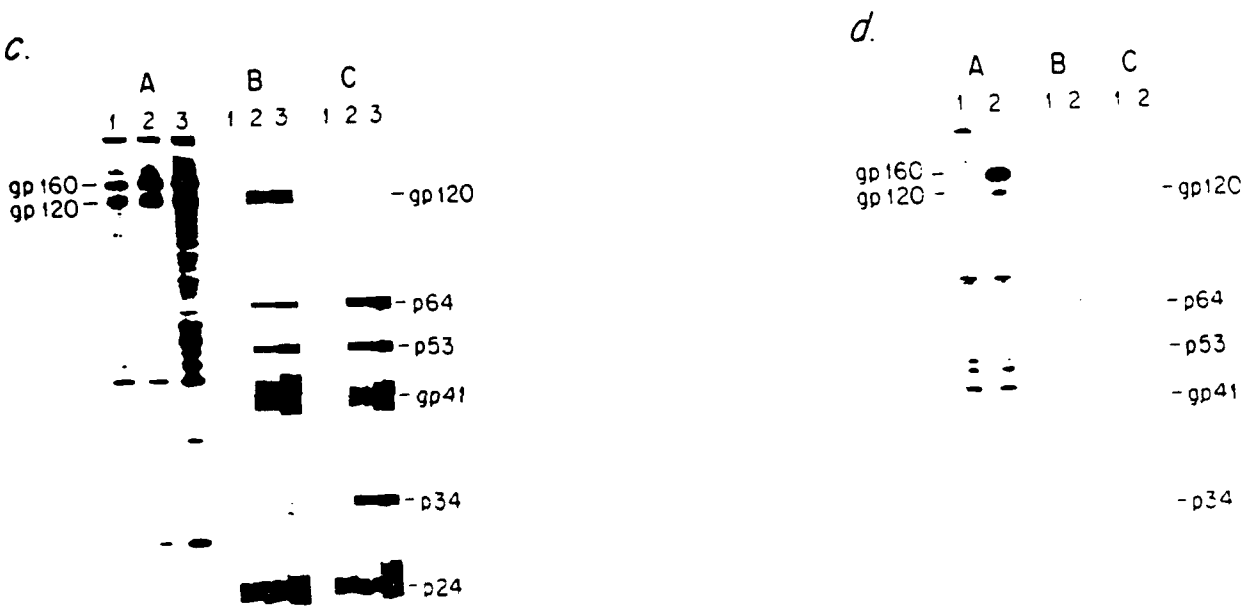


Progression in Seroreactivity Patterns. Four representative hemophiliacs with all of the available serial serum samples are illustrated in panels a to d. Each specimen was analyzed by three procedures: RIP (a), western blotting NR (b), and R (c).

Panel a: three samples from the first patient; the first serum dated April 1982 presenting pattern 1, the second serum dated Nov. 1983 presenting pattern 4, and the third serum dated Feb. 1986 presenting pattern 8.

Panel b: two samples from the second patient; the first serum dated Sept. 1981 presenting pattern 2, and the second serum dated April 1985 presenting pattern 5.

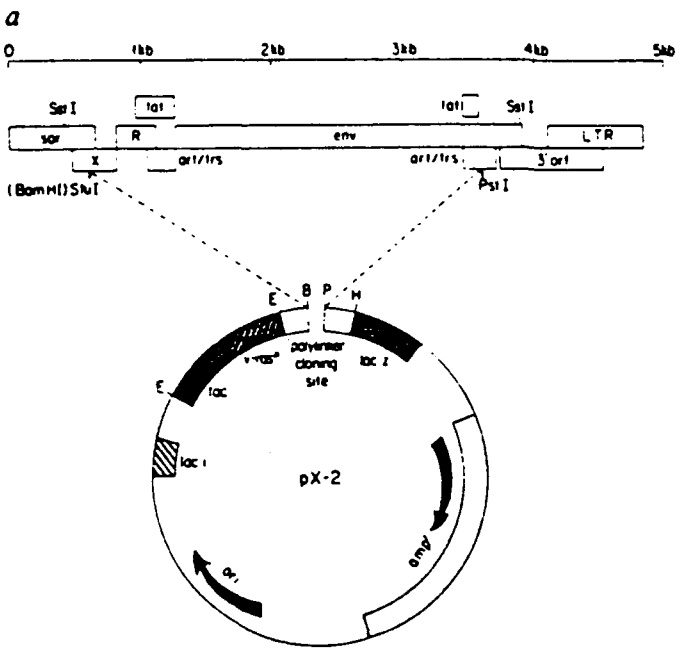
Figure 1



Panel c: three samples from the third patient; the first serum dated Oct. 1981 presenting pattern 3, the second serum dated Dec. 1983 presenting pattern 5, and the third serum dated March 1986 presenting pattern 8.

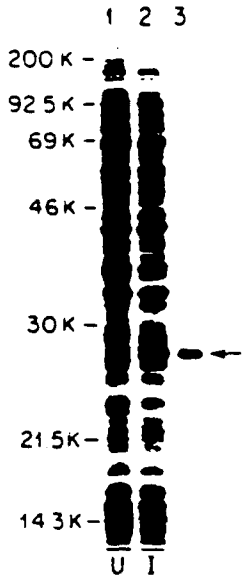
Panel d: two samples from the fourth patient; the first serum dated Dec. 1984 was negative and the second serum dated Oct. 1985 presented pattern 6.

Figure 2



(a). Bacterial expression plasmid pX-2. This plsmid is modified from plasmid pUC-18 and contains part of plasmids pXVR (17) and pBK-28 (18-19). It contains an EcoR I fragment which has a tac promoter linked to the first 333 nucleotides of v-ras^{H}. Downstream from the v-ras^{H} coding sequence is the pUC-18 polylinker cloning site. The 3.5 kb Sst I fragment of pBK-28 was excised and subcloned into pUC-18. This subcloned plasmid was digested with Stu I and ligated with a Bam HI linker, d(CGGGATCCCG). Plasmid DNA with the Bam HI linker was digested with Bam HI and Pst I. The 3.1 kb Bam HI-Pst I fragment which includes most of the vpx coding sequence (formerly called orf-x) was inserted into the polylinker cloning site.

Figure 2



b

333 bps from v-ras^H

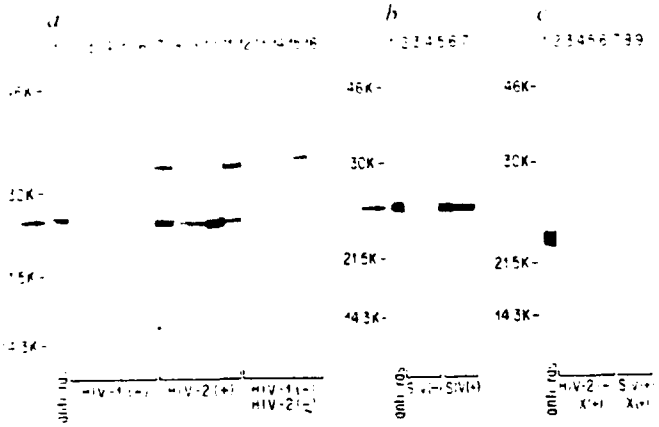
```
ATG              CCC ATG AAT TCG AGC TCG GTA CCC GGG GAT CCC
GCC TTC GAA TGG CTA AAC AGA ACA GTA GAG GAG ATA AAC AGA GAG
GCA GTA AAC CAC CTA CCA AGG GAG CTG ATT TTC CAG GTT TGG CAA
AGG TCT TGG GAA TAC TGG CAT GAT GAA CAA GGG ATG TCA CAA AGC
TAT GTA AAA TAC AGA TAC TTG TGT TTA ATG CAA AAG GCT TTA TTT
ATG CAT TGC AAG AAA GGC TGT AGA TGT CTA GGG GAA GGA CAC GGG
GCA GGA GGA TGG AGA CCA GGA CCT CCT CCT CCT CCC CCT CCA GGA
CTA GCA TAA
```

Sequence from orf - s

(b). DNA sequence analysis of pX-2. The nucleotide sequence of a 0.6 kb Hind III DNA fragment begining in the v-ras^H gene and ending in the R region of pBK-28 was determined by the dideoxy nucleotide sequencing method (USB, Cleveland, Ohio).

(c). Polyacrylamide gel electrophoresis of recombinant vpx protein. Lane 1: uninduced whole bacterial lysates (U); lane 2: isopropyl-ß-D-thiogalactopyranoside (IPTG) induced bacterial lysates (I); lane 3: partially purified vpx recombinant protein as indicated by the arrow.

Figure 3



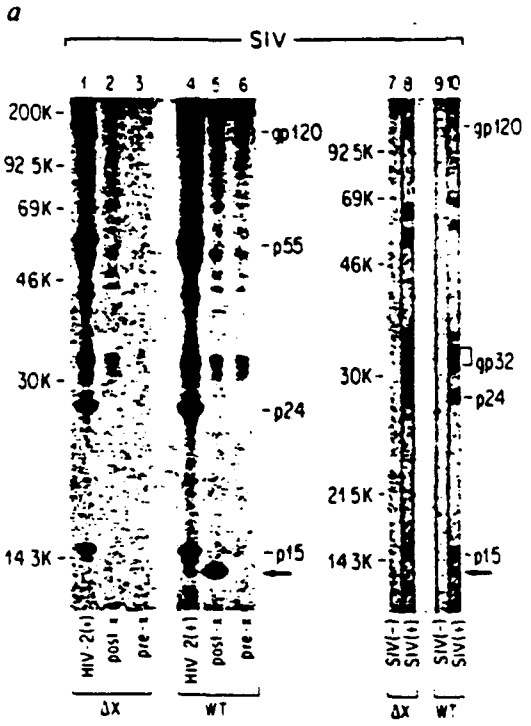
Immunoblotting of partially purified vpx recombinant protein (a and b).

(a). Lane 1: goat anti-ras serum; lanes 2-6: sera from HIV-1 positive donors; lanes 7-11: sera from HIV-2 positive West Africans; lanes 12-16: sera from blood donors seronegative for both HIV-1 and HIV-2.

(b). Lane 1: goat anti-ras serum; lanes 2-4: SIV seronegative monkey sera; lanes 5-7: SIV seropositive monkey sera. The arrow indicates 27 K vpx recombinant protein.

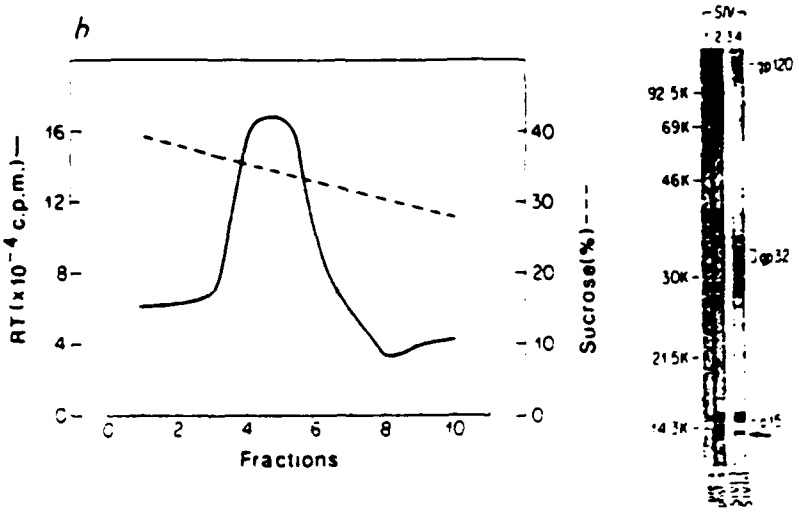
(c). Immunoblotting of partially purified ras protein. Lane 1: goat anti-ras serum; lanes 2-6: the same HIV-2 positive human sera shown in lanes 7-11 of Figure 3a; lanes 7-9: the same SIV positive sera shown in lanes 5-7 of Figure 3b.

Figure 4.



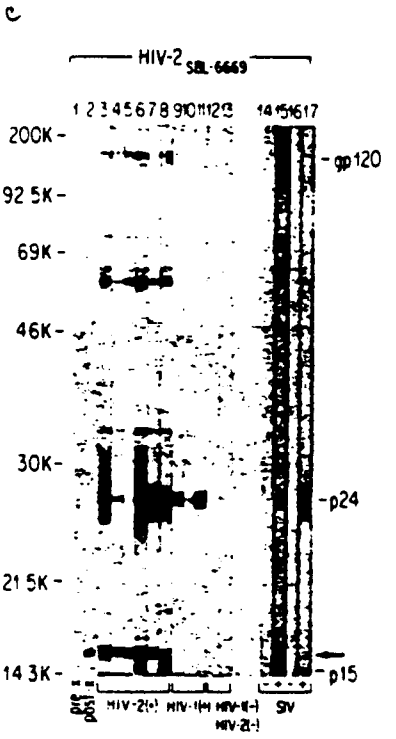
(a) Detection of $p12^x$ in SIV_{MAC} infected Hut-78 cells by radio-immunoprecipitation (RIP) and immunoblotting. RIP analysis: lanes 1-3: cell lysates from cultures infected by the vpx truncated mutant virus (Dx); lanes 4-6: cell lysates from cultures infected by the wild type virus (WT); lanes 1 and 4: serum from an HIV-2 positive donor; lanes 2 and 5: post-immune goat serum to the recombinant vpx protein; lanes 3 and 6: pre-immune serum from the same goat. The arrow indicates $p12^x$. Immunoblotting: lanes 7-8: viral lysates from cultures infected by the vpx truncated mutant virus; lanes 9-10: viral lysates from cultures infected by the wild type virus; lanes 7 and 9: SIV negative monkey sera; lanes 8 and 10: SIV positive monkey sera.

Figure 4.



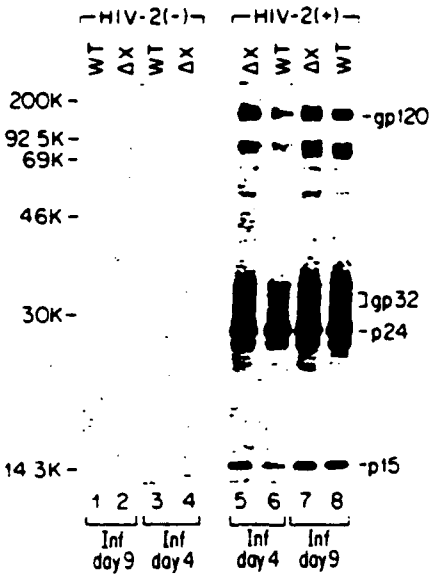
(b). Detection of $p12^x$ in sucrose gradient banded SIV_{MAC}. Left: reverse transcriptase activity of sucrose gradient banded SIV_{MAC}. Right: immunoblotting of SIV_{MAC} pooled from fractions 4 and 5. Sera tested were: lane 1: pre-immune goat serum; lane 2: post-immune goat serum to the recombinant vpx protein; lane 3: SIV seronegative monkey serum; lane 4: SIV seropositive monkey serum. The arrow indicates $p12^x$. Background binding to p24 and p15 by the pre-immune goat serum was observed.

Figure 4.



(c). Immunoblotting of HIV-$2_{SBL-6669}$ vpx protein. Lanes 1-17: viral lysates prepared from cell-free culture supernant of HIV-$2_{SBL-6669}$ producer cells. Lane 1: pre-immune goat serum; lane 2: post-immune goat serum to the recombinant vpx protein; lanes 3-8: sera from HIV-2 positive donors; lanes 9-11: sera from HIV-1 positive blood donors; lanes 11-13: sera from HIV-2/HIV-1 seronegative blood donors; lanes 14 and 16: sera from SIV negative monkeys; lanes 15 and 17: sera from SIV positive monkeys.

Figure 5



Immunoblotting of virion proteins produced by the wild type virus or the vpx mutant virus infected Hut-78 cells. Lanes 1 and 8: viral lysates from the wild type virus infected Hut-78 cells (day 9); lanes 2 and 7: viral lysates from the vpx mutant virus infected Hut-78 cells (day 9); lanes 3 and 6: viral lysates from wild type virus infected Hut-78 cells (day 4). Sera used were: lanes 1-4: serum from an HIV-1 and HIV-2 negative blood donor; lanes 5-8: pooled sera of six HIV-2 positive donors from West Africa.

Literature Cited

1. Chou MJ, et al. J Infect Dis 1988; **157**: 805-811.
2. Lee TH, et al. 1988. In *Vaccines 88 New chemical and genetic approaches to vaccination: Prevention of AIDS and other viral, bacterial, and parasitic diseases* (ed. Ginsberg H, et al.), p. 373-377. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
3. Lee TH, et al. 1989. *In Vaccines 89 Modern approaches to new vaccines including prevention of AIDS* (ed. Lerner RA, et al) , p. 191-194. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
4. Zagury D, et al. Nature 1988; **332**: 728.
5. Kitchen LW, et al. Nature 1984; **312**: 367-9.
6. Laemmli UK. Nature 1970; **227**: 680-5.
7. Barin F, et al. Lancet 1985; **2**: 1387-9.
8. Wain-Hobson S, et al. Cell 1985; **40**: 9-17.
9. Ratner L, et al. Nature 1985; **313**: 277-284.
10. Sanchez-Pescador R, et al. Science 1985; **227**: 484-492.
11. Muesing MA, et al. Nature 1985; **313**: 450-58.
12. Arya SK, et al. Science 1985; **229**: 69-73.
13. Sodroski J, et al. Science 1985; **229**: 74-77.
14. Sodroski J, et al. Nature 1986; **321**: 412-17.
15. Feinberg MB, et al. Cell 1986; **46**: 807-17.
16. Matsuda Z, et al. Proc Natl Acad Sci U.S.A. 1988; **85**: 6968-72.
17. Feig LA , et al. Proc Natl Acad Sci U.S.A. 1986; **83**: 4607-4611.
18. Kornfeld H, et al. Nature 1987; **326**: 610-613.
19. Kestler HW III, et al. Nature 1988; **331**: 619-621.
20. Albert J, et al. AIDS Research and Human Retroviruses 1987; **3**: 3-10.
21. Franchini G, et al. AIDS Research and Human Retroviruses 1987; **3**: 11-17.
22. Pallas DC, et al. J Virol 1986; **60**: 1075-1084.

23. Lee TH, et al. Proc Natl Acad Sci U.S.A. 1984; **81**: 3856-3860.

24. Rho HM, et al. Virology 1981; **112**: 355-360.